Confirmation of functional hermaphroditism in six grouper species (Epinephelidae: Epinephelinae) from the Gulf of Mexico

by

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Received: 14 Apr. 2015
Accepted: 24 Sep. 2015
Editor: K. Rousseau

Abstract. – Diagnosing hermaphroditism in teleost fish requires careful assessment and is particularly challenging in groupers. Established, reliable criteria to detect functional protogynous hermaphroditism were used to collect data on sexual pattern in six minimally studied grouper populations on the continental shelf of the Yucatan Peninsula. Individuals in the process of changing sex (transitional individuals), considered strongly indicative of protogyny, were observed. Functional protogynous hermaphroditism was confirmed for *Epinephelus morio* (Valenciennes, 1828) and *Mycteroperca venenosa* (Linnaeus, 1758), and for populations of *Epinephelus guttatus* (Linnaeus, 1758), *Mycteroperca bonaci* (Poey, 1860) and *Mycteroperca tigris* (Valenciennes, 1833) from the southern Gulf of Mexico. Although *Mycteroperca microlepis* (Goode & Bean, 1880) has been confirmed as protogynic in previous studies, distinctive transitional individuals were not identified in the analysed sample. Transitional individual rates were low in the studied sample: 1.0% for *E. morio*; 0.5% for *E. guttatus* and *M. bonaci*; 0.6% for *M. tigris*; and 0.2% for *M. venenosa*. Timing of sex change in these grouper species did not exhibit any seasonal pattern. For *E. morio*, *M. bonaci* and *M. microlepis*, the lack of primary males or bisexual individuals among juveniles collected from inshore waters, and the presence of morphological features characteristic of secondary males in testes from adults collected from offshore waters, suggest monandry in the southern Gulf of Mexico populations of these three species.

Key words

Serranidae
Epinephelus sp.
Mycteroperca sp.
Gulf of Mexico
Reproduction
Hermaphroditism

Résumé. – Confirmation de l'hermaphrodisme fonctionnel chez six espèces de mérou (Epinephelidae: Epinephelinae) du golfe du Mexique.

Le diagnostic de l'hermaphrodisme chez les poissons exige une évaluation soigneuse et est particulièrement difficile à réaliser chez les mérous. Les critères les plus sûrs pour détecter l'hermaphrodisme protogyne fonctionnel chez les poissons ont été utilisés dans le but d'accroitre les connaissances sur le type de sexualité que présentent six espèces de mérous originaires de populations peu étudiées de la plateforme continentale de la péninsule du Yucatan. Des individus en cours de changement de sexe (individus en inversion sexuelle), considérés comme un critère robuste pour l'identification de la protogynie, ont été observés. Le diagnostic de l'hermaphrodisme protogyne fonctionnel a donc été confirmé chez *Epinephelus morio* (Valenciennes, 1828) et *Mycteroperca venenosa* (Linnaeus, 1758) ainsi que chez les populations d'*Epinephelus guttatus* (Linnaeus, 1758), de *Mycteroperca bonaci* (Poey, 1860) et de *Mycteroperca tigris* (Valenciennes, 1833) du sud du golfe du Mexique. Bien que la protogynie ait été confirmée dans des études antérieures pour *Mycteroperca microlepis* (Goode & Bean, 1880), il n'a pas été possible d'identifier des individus en inversion sexuelle dans la population de cette espèce du sud du golfe du Mexique. Lorsque des individus en inversion sexuelle sont observés, ceux-ci sont présents en faibles pourcentages dans les échantillonnages: 1,0% pour *E. morio*; 0,5% pour *E. guttatus* et *M. bonaci*; 0,6% pour *M. tigris* et 0,2% pour *M. venenosa*. La période à laquelle se produit le changement de sexe chez ces espèces n'est généralement pas liée à un cycle saisonnier. Pour *E. morio*, *M. bonaci* et *M. microlepis* l'absence de mâles primaires et d'individus bisexuels chez les juvéniles collectés dans les eaux côtières et la présence de structures morphologiques caractéristiques des mâles secondaires dans les testicules d'adultes collectés en eaux profondes, laissent supposer que les populations de ces trois espèces du sud du golfe du Mexique sont monandriques.

Diagnosis of hermaphroditism in teleost fish requires careful assessment. A population or species is considered to be functionally hermaphroditic if at some point(s) during its life cycle, a substantial proportion of individuals functions as both sexes, either simultaneously, sequentially (sex changers), or serially (bidirectional and cyclical sex changers). Sequential hermaphroditism, especially protogyny (i.e. a functional female to a functional male sex change) is particularly common in shallow tropical marine reef habitats and among the perciforms. Protogyny generally occurs in two forms. In the first, all males are derived from functional females (*i.e.* secondary males) in what is called monandry, or a single pathway of male development. In the second, some males are male at first maturation (i.e. primary males), while others arise from a sex change in functional females (secondary males); this is called diandry, or two pathways of male development (Sadovy and Shapiro, 1987; Sadovy de Mitcheson and Liu, 2008).

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Cybium 2016, 40(1): 83-92.

In the past, protogyny has been diagnosed using at least ten different gonad histology and population structure features in various combinations (Shapiro, 1987). However, many of the criteria used in earlier studies have been reconsidered in the light of new findings. Two features are currently considered strongly indicative of protogyny: 1) detailed gonadal histological series showing various stages of sexual transition; and 2) field or laboratory observations of functional sex change in identified individuals. Definitive evidence for protogyny via histological examination of gonads is confirmed by identification of transitional individuals with gonads containing degenerating ovarian tissue and proliferating testicular tissue; and/or secondary males with testes containing sperm sinuses within the gonadal capsule and a remnant of the former ovarian membrane-lined central cavity not used for sperm transport. Yellow bodies and oocytes in testes are non-specific features. If used alone, population structure features such as bimodal size-frequency distributions (with males larger than females), or sex-ratios differing from unity are not reliable indicators of sexual pattern (Sadovy and Shapiro, 1987; Sadovy de Mitcheson and Liu, 2008).

Protogyny is a salient feature of grouper (Epinephelidae: Epinephelinae; Smith and Craig, 2007) reproduction and is confirmed in the genera *Cephalopholis*, *Epinephelus*, *Mycteroperca* and *Plectropomus* (Sadovy and Shapiro, 1987; Sadovy de Mitcheson and Liu, 2008). However, confirmation of sexual pattern in groupers is rather challenging. In a number of species, some testes are secondary in configuration but do not necessarily pass through a functional female phase (e.g. *Epinephelus andersoni* Boulenger, 1903; Fennessy and Sadovy, 2002). Consequently, testicular morphology alone is not always strongly indicative of protogyny in groupers. In this case, detection of transitional individuals in natural populations in conjunction with experimental induction of sex change in the field or laboratory, when possible, are the only reliable indicators for detecting protogyny. It is also quite often difficult to distinguish diandry from monandry in groupers based solely on testicular morphology analysis (Sadovy de Mitcheson and Liu, 2008).

Twenty-one grouper species are known in the waters of Mexico. They are the most important demersal finfish resource exploited in the southern Gulf of Mexico, particularly on the continental shelf of the Yucatan Peninsula (Campeche Bank) (SAGARPA, 2012). In this region, grouper stocks are currently considered fully exploited, and certain species are overexploited, such as red grouper *Epinephelus morio* (Valenciennes, 1828), the most heavily captured species (Fernández *et al*., 2010). Except for *E. morio*, the status of other specific grouper stocks remains unknown. Adequate management of any grouper fishery involves developing precautionary measures to prevent depletion, for which data on the sexual pattern of each grouper species is critical. Hermaphroditic populations may respond differently to fishing

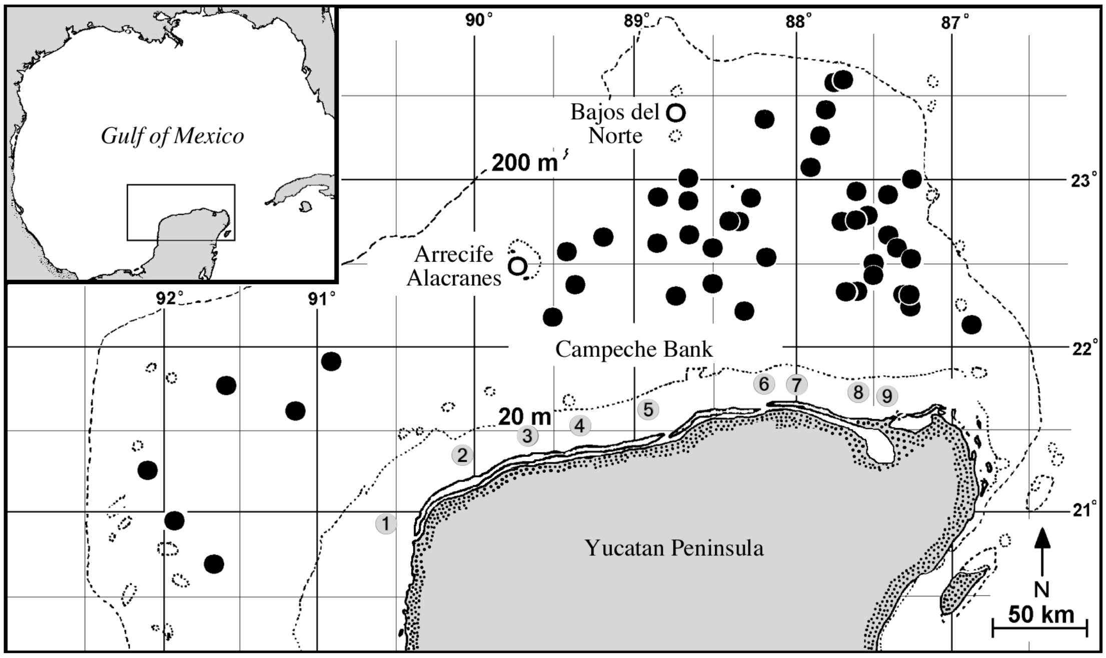

Figure 1. - Sample collection areas for *Epinephelus morio*, *Mycteroperca bonaci* and *Mycteroperca microlepis* in inshore (●) and offshore (●) waters, and for *Epinephelus guttatus*, *Mycteroperca tigris* and *Mycteroperca venenosa* at (○) Arrecife Alacranes (22º21'44"N to 22º35'12"N; 89º36'30"W to 89º48'00"W) and (○) Bajos del Norte (23º20'N; 88º45'W), on Campeche Bank, southern Gulf of Mexico. Inshore water sites were: 1: Celestún; 2: Sisal; 3: Progreso; 4: Telchac Puerto ; 5: Dzilam de Bravo; 6: San Felipe; 7: Río Lagartos; 8: El Cuyo; 9: Punta Caracol.

pressure than gonochoristic ones (Bannerot *et al.*, 1987). Protogyny poses a particular problem for rational fishery management. On average, grouper males tend to be larger than females, creating the possibility that size-selective fishing mortality results in differential loss of larger males. Consequently, an insufficient number of males may remain in the reproductive population to fertilize eggs from females, causing an overall reduction in a population's reproductive output (Shapiro, 1987; Sadovy, 1996; Coleman *et al.*, 1999).

The present study objective was to generate sexual pattern data for six commonly captured but minimally studied grouper populations on Campeche Bank, Mexico: the rocky-bottom species *E. morio*, black grouper *Mycteroperca bonaci* (Poey, 1860) and gag *Mycteroperca microlepis* (Goode & Bean, 1880); and the coral-reef species rock hind *Epinephelus guttatus* (Linnaeus, 1758), tiger grouper *Mycteroperca tigris* (Valenciennes, 1833) and yellowfin grouper *Mycteroperca venenosa* (Linnaeus, 1758).

MATERIALS AND METHODS

Sample collection

Data were obtained from different areas of the broad shallow carbonate continental shelf of the Yucatan Peninsula, known as Campeche Bank (Fig. 1). Water characteristics in the region are influenced by a seasonal upwelling, which produces a dynamic uplift of the Caribbean Subtropical Underwater (CSUW) along its eastern margin that reaches maximum intensity between April and June (Merino, 1997). As a consequence, mean bottom water temperature on Campeche Bank is lower in summer (20.5°C in July) than in winter (24°C in December and January) (Piñeiro *et al.*, 2001). During summer, a salty (~36.5) and warm (> 26°C) water mass is present in the coastal zone of the Yucatan and forms a strong stratification with the CSUW, which remains in the East and offshore, close to the bottom (Enriquez *et al.*, 2013). Campeche Bank represents one of the main commercial exploitation areas for groupers in the Gulf of Mexico (Stevenson, 1981).

Grouper specimens were collected monthly from fishery-independent or -dependent catches, during several time periods between August 1988 and October 2009 (Tab. I). Since *E. morio*, *M. bonaci* and *M. microlepis* are depth-size distributed, specimens of these species were collected from rocky bottom in both offshore (30-210 m depth) and coastal inshore (1-27 m depth) waters of Campeche Bank. Specimens of the reef-associated *E. guttatus*, *M. tigris* and *M. venenosa* were collected at two coral reefs north of the Yucatan coast: Arrecife Alacranes (15-27 m depth; 130 km from the coast) and Bajos del Norte (10-18 m depth, 254 km from the coast). Arrecife Alacranes is a large emergent coral-reef system, and Bajos del Norte a submerged bank reef. *Epinephelus guttatus* forms spawning aggregations at both reefs while *M. tigris* and *M. venenosa* do so only at Bajos del Norte (Tuz-Sulub and Brulé, 2015). Fishing gear included long-lines for offshore catches, and hook-and-line, spear guns or trawl nets for inshore and coral reef catches.

Fish and gonad analysis

For all the collected fish, measurements were taken of body length at the caudal fork (FL; nearest 0.1 cm), whole

Table I. - Number, sex and size range of six grouper species collected between August 1988 and October 2009 on the continental shelf of the Yucatan Peninsula (Campeche Bank). [a]: Fishery-independent collections; [b]: Fishery-dependent collections; [*]: Specimens with gonads containing both ovarian and testicular tissue in varying proportions (presumed transitional individuals).

			Sex					
Species	Collection		Female		Female/male[*]		Male	
	Area	Date	N	Size range (cm FL)	N	Size range (cm FL)	N	Size range (cm FL)
E. guttatus	Arrecife Alacranes[a]	Feb. 2008-Oct. 2009	216	20.5-48.5	3	21.1-35.5	191	26.3-49.0
	Bajos del Norte[b]		485	24.5-51.0	6	39.5-50.0	889	28.0-57.5
E. morio	Inshore waters[a]	Aug. 1988-Sep. 1993	329	12.3-55.5	0	–	0	–
	Offshore waters[b]	Jun. 1989-Apr. 1992	676	38.9-85.4	11	50.9-72.7	199	41.0-88.0
		Apr. 1996-Jan. 1998	270	39.0-80.0	4	51.0-62.5	139	44.1-89.0
		Feb-Mar. 2003	195	32.0-78.8	3	40.8-52.5	57	35.5-80.0
M. bonaci	Inshore waters[a]	Nov. 1998-May. 1999	39	25.6-58.0	0	–	0	–
	Offshore waters[b]	Apr. 1996-May. 1999	661	57.0-123.5	5	99.0-121.5	214	86.0-132.0
M. microlepis	Inshore waters[a]	Dec. 1999-Dec. 2001	107	9.0-49.0	0	–	0	–
	Offshore waters[b]	Apr. 1996-Apr. 1999	161	49.5-111.0	2	85.0; 96.0	49	95.0-116.0
M. tigris	Arrecife Alacranes[a]	Feb. 2008-Oct. 2009	82	19.5-61.5	0	–	4	55.0-75.0
	Bajos del Norte[b]		243	35.5-71.1	7	45.0-67.5	289	38.0-81.0
M. venenosa	Arrecife Alacranes[a]	Feb. 2008-Oct. 2009	231	19.8-102.0	0	–	4	34.0-89.0
	Bajos del Norte[b]		1222	34.4-94.0	4	44.0-76.0	157	42.5-98.5

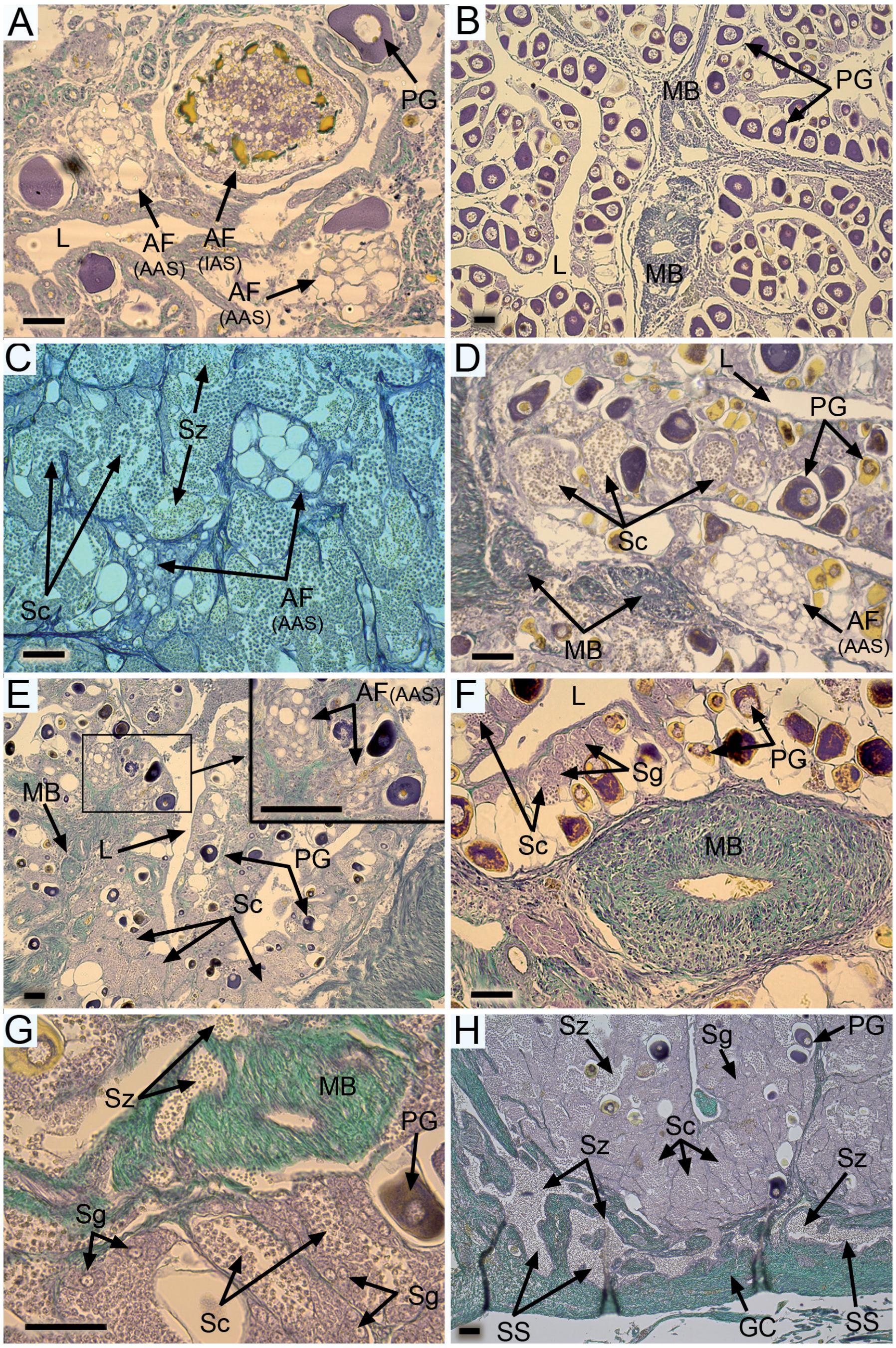

Figure 2. - Histological sections of gonads from five groupers from Campeche Bank (Gabe and Martoja's one-step trichrome stain). **A**: *Mycteroperca venenosa*, female in regressing reproductive phase (71.5 cm FL), collected April 2009 at Bajos del Norte, showing atretic follicles in intermediate and advanced atretic stages. **B**: *Mycteroperca microlepis*, female in regenerating reproductive phase (97.0 cm FL), collected July 1998 in offshore waters, showing muscle bundles. **C**: *Epinephelus guttatus*, transitional individual (50.0 cm FL), collected February 2009 at Bajos del Norte, showing atretic follicles in advanced atretic stage and crypts of spermatocytes and spermatozoa. **D**: *Epinephelus morio*, transitional individual (40.5 cm FL), collected February 2003 in offshore waters, showing remnant of the former ovarian lumen, atretic follicles in advanced atretic stage, muscle bundles and crypts of spermatocytes. **E**: *Mycteroperca tigris*, transitional individual (48.2 cm FL), collected June 2008 at Bajos del Norte, showing remnant of the former ovarian lumen, atretic follicles in advanced atretic stage, muscle bundles and crypts of spermatocytes. **F**: *Mycteroperca bonaci*, transitional individual (121.5 cm FL), collected December 1997 in offshore waters, showing remnant of the former ovarian lumen, muscle bundle and crypts of spermatogonia and spermatocytes. **G-H**: *Mycteroperca venenosa*, transitional individual (55.0 cm FL), collected January 2009 at Bajos del Norte, showing muscle bundle and crypts of spermatogonia, spermatocytes and spermatozoa (G); and sperm sinus within gonad capsule (H). AF: atretic follicle; AAS: advanced atretic stage; IAS: intermediate atretic stage; GC: gonadal capsule; L: lumen; MB: muscle bundle; PG: primary growth oocyte; Sc: spermatocyte; Sg: spermatogonia; SS: sperm sinus; Sz: spermatozoa. Scale bars = 50 μm.

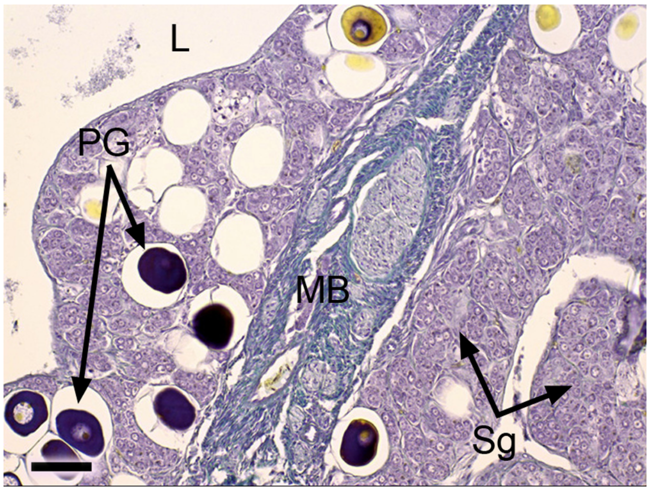

Figure 3. - Histological sections of gonads of *Mycteroperca microlepis* individual (85.0 cm FL), collected May 1996 in offshore waters of the Campeche Bank (Gabe and Martoja's one-step trichrome stain). Individual most likely male, showing remnant of the former ovarian lumen, numerous crypts of spermatogonia, and some muscle bundles and remnant primary growth oocytes. L: lumen; MB: muscle bundle; PG: primary growth oocyte; Sg: spermatogonia. Scale bar = 50 μm.

body weight (WW; nearest 1 g), gutted weight (GW; nearest 1 g), and fresh gonad weight (gW; nearest 0.01 g). A gonad fragment from each individual was prepared for light microscopy. In *Epinephelus* and *Mycteroperca* individuals, the entire gonad is an admixture of ovarian and testicular tissue (*Epinephelus* type). The presumptive testicular tissue is scattered in the form of crypts throughout the epithelial layer of the ovarian lamellae (Smith, 1965). A piece of a transverse section from the median portion of one gonad from each specimen was removed and preserved in Bouin fluid for six to seven days. Fixed gonad samples were then dehydrated and embedded in paraffin or Paraplast. Four to five histological sections per gonad were thin-sectioned (6 μm) and stained in Gabe and Martoja's one-step trichrome stain (Gabe, 1968). Histological slides were viewed on a Leica DM2700 M RL/TL microscope and gonad section images taken with a Leica MC1170 HD camera and processed with the Leica LAS software.

Sex (male, female or transitional) and gonad developmental stage were recorded for each specimen by microscopic examination of gonad structure. The criteria used to define sexual pattern in the six studied grouper species were those proposed by Sadovy and Shapiro (1987) and updated by Sadovy de Mitcheson and Liu (2008) for characterizing hermaphroditism in teleost fish. Histological sections of gonads were scanned to detect transitional individuals in protogynous species; these were defined as individuals with gonads containing degenerating ovarian tissue or muscle bundles and proliferating testicular tissue. Degenerating ovarian tissue in gonads is characterized by the presence of atretic follicles (i.e. atretic vitellogenic oocytes) (Fig. 2A). Muscle bundles are blood vessels surrounded by connective and muscle tissue, located in the centre region of the ovarian lamellae. These structures are indicative of prior spawning as a female, and become evident after expansion and subsequent post-spawning collapse of the ovarian lamellae (Fig. 2B). Proliferating testicular tissue in gonads is characterized by the presence of crypts or clusters of spermatocytes, spermatids or spermatozoa (Sadovy and Shapiro, 1987; Shapiro *et al*., 1993; Fennessy and Sadovy, 2002; Brown-Peterson *et al*., 2011). Histological examination also allowed calculation of the proportions of males with testes containing a previous ovarian membrane-lined central cavity (i.e. lumen), sperm sinuses within gonadal capsule and remnant oocytes. The gametogenesis stages used for males were based on Moe (1969), while those for females were based on Wallace and Selman (1981). Gonadal development was analyzed based on the reproductive phases defined by Brown-Peterson *et al*. (2011). The individual gonadosomatic index [GSI = 100 * (gW/GW)] was calculated.

Statistical analysis

Analyses were also done of mean or median sizes in males and females, size-frequency distribution by sex, and sex ratio. A one-sized test or a Mann-Whitney nonparametric test were applied to compare mean or median sizes between males and females, and a Kolmogorov-Smirnov nonparametric test was applied to compare size frequency distributions by sex. Sex ratio was calculated using the number of females to each male (F:M), excluding individuals in sexual transition. A Pearson chi-square goodness-of-fit test was applied to determine if the calculated sex ratio differed from the balanced sex ratio (1:1). Nonparametric statistics were used when mean and standard deviation based on normal theory did not apply. Statistical analyses were run with SYSTAT Statistics version 8.0 (www.systat.com) or INFOSTAT (www.infostat.com.ar), with an α of 0.05.

RESULTS

Among the six studied species (N_{total} = 7,154), 45 individuals had gonads containing both ovarian and testicular tissue: nine for *E. guttatus*; 18 for *E. morio*; five for *M. bonaci*; two for *M. microlepis*; seven for *M. tigris*; and four for *M. venenosa* (Tab. I). All these gonads exhibited a distinctive ovarian structure. The lumen was lined with germinal epithelium forming the surface layers of a series of lamellae. Ovarian and testicular tissues in diverse developmental stages were scattered in varying proportions inside the lamellae, surrounded loosely by connective tissue and blood vessels. Primary growth oocytes were always present in these gonads, except in one *E. guttatus* specimen (50.0 cm

Table II. - Histological features, size range and month of capture for specimens of six grouper species collected on Campeche Bank between August 1988 and October 2009, with gonads containing both ovarian and testicular tissue in varying proportions. PG oocytes: primary growth oocytes; Sg: spermatogonia; Sc: spermatocytes; St: spermatids; Sz: spermatozoa. *: Specimens not considered as transitional individuals. +: observed; (+): observed in most individuals; -: not observed.

	Histological features											
	Ovarian tissue		Muscle bundles	Testicular tissue				Gonadal structure		N		
				Crypts								
Species	PG oocytes	Atretic follicles		Sg	Sc	St	Sz	Ovarian-like lumen	Sperm sinuses		Fork length range (cm)	Month of capture
E. guttatus	(+)	+	+	+	+	+	+	+	-	4	39.5-50.0	Feb.; Apr.; May
	+	-	+	+	+	+	+	+	-	4	32.0-43.1	May; Jun.; Oct.
	+	-	-	+	-	-	-	+	-	1^*	21.1	Jan.
E. morio	+	+	+	+	+	+	+	+	-	5	40.5-59.4	Feb.; Dec.
	+	-	+	+	+	+	+	+	(+)	10	40.8-72.7	Jan.; Feb.; Jun.; Aug.; Dec.
	+	-	-	+	+	-	+	+	-	3^*	50.9-53.0	Jun.; Aug.
M. bonaci	+	-	+	+	+	+	+	+	+	4	99.0-121.5	Jan.; Mar; Nov.; Dec.
	+	-	+	+	-	-	-	+	-	1^*	103.0	Sep.
M. microlepis	+	-	+	+	-	-	-	+	-	2^*	85.0; 96.0	May; Jun.
M. tigris	+	+	+	+	+	-	-	+	-	1	48.2	Jun.
	+	-	+	+	+	-	-	+	-	2	54.0-67.0	Sep.; Oct.
	+	-	+	+	-	-	-	+	-	3^*	46.0-49.0	Aug.; Sep.
	+	-	-	+	+	-	-	+	-	1^*	40.5	Jun.
M. venenosa	+	-	+	+	+	+	+	+	+	3	44.0-76.0	Jan.; Sep.; Dec.
	+	-	+	+	-	-	-	+	-	1^*	69.5	Aug.

FL). Ten individuals (four *E. guttatus* [GSI range = 0.35-1.04%]; five *E. morio* [GSI = 0.26-0.53%]; and one *M. tigris* [GSI = 0.16%]) had gonads containing atretic follicles, muscle bundles and crypts of spermatogonia, spermatocytes and eventually spermatids and spermatozoa (Tab. II). In all cases, the atretic follicle stage observed in the gonads was characterized by large intercellular cavities (Fig. 2C, D, E). Twenty-three specimens (ten *E. morio* [GSI = 0.12-0.47%]; four *E. guttatus* [GSI = 0.06-0.29%]; four *M. bonaci* [GSI = 0.04-0.21]; three *M. venenosa* [GSI = 0.12%; N = 1]; and two *M. tigris* [GSI = 0.18% and 0.23%]) had muscle bundles and crypts of spermatogonia, spermatocytes, and eventually spermatids and spermatozoa, in their gonads (Fig. 2F, G; Tab. II). Sperm sinuses within the gonadal capsule could be observed in three of these individuals (Fig. 2H): one *E. morio* (72.7 cm FL); one *M. bonaci* (99.0 cm FL); and one *M. venenosa* (55.0 cm FL). The remaining twelve fish (four *M. tigris* [GSI = 0.13-0.17%]; three *E. morio* [GSI = 0.07-0.11%]; two *M. microlepis* [GSI = 0.05 and 0.20%]; one *E. guttatus* [GSI = 0.07%]; one *M. bonaci* [GSI = 0.28%]; and one *M. venenosa* [GSI = 0.35%]) had either muscle bundles with crypts of spermatogonia (Fig. 3) or only crypts of spermatogonia and spermatocytes in their gonads (Tab. II). These last twelve specimens cannot be considered transitional individuals because their gonads had either degenerating ovarian tissue without evidence of proliferating testicular tissue, or proliferating testicular tissue without evidence of degenerating ovarian tissue. With the exception of *M. microlepis*, the above results are strong evidence of the presence of transitional individuals in five of the studied species: *E. guttatus* ($N_{total} = 8$; $N_{ArrecifeAlacranes} = 2$ and $N_{Bajosdel\ Norte} = 6$); *E. morio* (N = 15); *M. bonaci* (N = 4); *M. tigris* (N = 3), and *M. venenosa* (N = 3).

Based on these data, the overall rate of transitional individuals in the study sample from Campeche Bank was 0.5% for *E. guttatus* (0.5% at Arrecife Alacranes and 0.4% at Bajos del Norte); 1.0% for *E. morio* and 0.5% for *M. bonaci* (offshore waters); and 0.6% for *M. tigris* and 0.2% for *M. venenosa* (Bajos del Norte). Depending on the species, transitional individuals were observed in collections during three (*M. venenosa*) to seven (*E. morio*) months of the year (Tab. II).Transitional individuals never occurred in inshore waters of Campeche Bank; only juvenile *E. morio*, *M. bonaci* and *M. microlepis* females were observed in this area. Neither *M. tigris* nor *M. venenosa* were observed to aggregate for spawning at Arrecife Alacranes, and no transitional individuals of these two species were caught at this site (Tab. I).

In all the examined groupers, a membrane-lined central cavity similar to that present in the ovaries was observed in the testes of most males (range: 60-100%). Sperm sinuses within the gonadal capsule were identified in 37% to 93% of males. In most of the studied species, primary growth oocytes were present in the testes of no more than 2.5% of the males. The notable exceptions were the presence of these remnant ovarian cells in 13% of *M. bonaci* males and 78% to 100% of *E. morio* males (Tab. III).

In all six species, mean or median FL was lower for females than for males, and male size range differed significantly from that of females. Generally, the overall F:M ratio was significantly biased in favor of females, although at Arrecife Alacranes *E. guttatus* exhibited a balanced sex ratio, and at Bajos del Norte more males than females were recorded for *E. guttatus* and *M. tigris* (Tab. IV).

Table III. - Percentage of males with testes showing strong (lumen and sperm sinuses) and weak (remnant PG oocytes) evidences for protogyny, for six grouper species collected on Campeche Bank. [1]: Arrecife Alacranes and Bajos del Norte collections; [2]: Offshore collections; N = number of males.

		Testis morphology			
Species	N	Lumen	Sperm sinuses	Remnant PG oocytes	Date of collection
E. guttatus[1]	1080	87%	77%	2.5%	Feb. 2008-Oct. 2009
E. morio[2]	199	100%	60%	78%	Jun. 1989-Apr. 1992
	139	100%	77%	86%	Apr. 1996-Jan. 1998
	57	100%	70%	100%	Feb.-Mar. 2003
M. bonaci[2]	214	76%	37%	13%	Apr. 1996-May 1999
M. microlepis[2]	49	88%	49%	2%	Apr. 1996-Apr. 1999
M. tigris[1]	289	88%	75%	1.4%	Feb. 2008-Oct. 2009
M. venenosa[1]	157	60%	93%	1.9%	Feb. 2008-Oct. 2009

DISCUSSION

In protogynous groupers, testicular tissue arises sporadically throughout the germinal epithelia of ovarian lamellae. Consequently, determining the functional sexual pattern in these fishes requires detection of gonads in transition between female and male function (Sadovy de Mitcheson and Liu, 2008). This essential criterion was clearly documented in five of the grouper species analysed here. Transitional individuals were unambiguously identified in populations of *E. guttatus*, *E. morio*, *M. bonaci*, *M. tigris* and *M. venenosa* from Campeche Bank. Gonads in transitional specimens contained atretic follicles and/or muscle bundles, both features indicative of prior female function (Sadovy and Shapiro, 1987; Shapiro *et al.*, 1993; Fennessy and Sadovy, 2002), intermixed with proliferating testicular tissue, including spermatocytes, spermatids and spermatozoa. Most of the atretic follicles in these gonads exhibited morphological characteristics similar to

Table IV. - Statistical analyses of specimens of six grouper species collected on Campeche Bank between June 1989 and October 2009: one-sized (Z) and Mann-Whitney (H) tests used to compare mean and median sizes between males and females; Kolmogorov-Smirnov (KS) test used to compare size frequency distributions by sex; and Pearson chi-square (χ^2) test used to determine if the calculated sex ratio differed from the balanced sex ratio (1:1). N: sample size; SD: standard deviation; F:M: number of females to each male. [1]: Arrecife Alacranes collections; [2]: Bajos del Norte collections; [3]: Offshore collections; [a]: June 1989-April 1992; [b]: April 1996-January 1998; [c]: February-March 2003. Bold italics indicate statistical significance ($p < 0.05$).

			Fork length (cm)					Sex ratio	
Species	Sex	N	Mean ± SD	Median	Z	$H_{df=1}$	KS	F:M	χ^2
E. guttatus	Female[1]	216	35.5 ± 4.9	35.5	-	***10925.5***	***0.375***	1.13:1.00	1.5
	Male[1]	191	39.5 ± 4.0	40.0					
	Female[2]	485	39.2 ± 4.1	39.0	-	***97547.0***	***0.429***	0.55:1.00	***118.8***
	Male[2]	889	43.2 ± 3.6	43.0					
E. morio[3]	Female[a]	676	59.9 ± 8.8	59.0	***4.24***	-	***0.288***	3.39:1.00	***260.0***
	Male[a]	199	67.3 ± 10.8	66.6					
	Female[b]	270	55.0 ± 8.2	53.5	-	***12571.5***	***0.237***	1.94:1.00	***42.0***
	Male[b]	139	61.9 ± 12.1	58.0					
	Female[c]	195	46.7 ± 9.5	43.5	-	***4165.0***	***0.284***	3.36:1.00	***74.2***
	Male[c]	58	52.4 ± 13.0	47.5					
M. bonaci[3]	Female	661	96.6 ± 12.1	98.0	***26.71***	-	***0.070***	3.27:1.00	***228.4***
	Male	214	114.6 ± 7.1	114.0					
M. microlepis[3]	Female	161	90.7 ± 10.3	93.0	***14.19***	-	***0.146***	3.29:1.00	***59.7***
	Male	49	104.7 ± 3.9	105.0					
M. tigris[2]	Female	243	48.9 ± 7.1	48.0	-	***116.0***	***0.420***	0.84:1.00	***4.0***
	Male	289	56.0 ± 7.3	56.0					
M. venenosa[2]	Female	1222	58.4 ± 8.8	58.0	-	***222.4***	***0.620***	7.78:1.00	***822.5***
	Male	157	73.7 ± 10.4	75.0	-				

follicles undergoing advanced atresia in the ovaries of the simultaneous hermaphrodite S*erranus cabrilla* (Linnaeus, 1758) (Febvre, 1975) and of other teleost species (Lambert, 1970; Miranda *et al.*, 1999). For each of these five species, transitional individuals had low GSI values similar to those found in immature females or mature females in regressing or regenerating reproductive phases (Brulé *et al.*, 1999, 2003a, b; Caballero-Arango, 2013; Caballero-Arango *et al.*, 2013). The highest GSI values were observed in *E. morio* (0.53%) and *E. guttatus* (1.04%), in two transitional individuals exhibiting gonads containing degenerating ovarian tissue (i.e. atretic follicles) and structures indicative of prior spawning as a female (i.e. muscle bundles).

Protogynous hermaphroditism was first proposed by Smith (1959) for *E. guttatus*, *M. bonaci*, *M. tigris* and *M. venenosa*; by McErlean and Smith (1964) for *M. microlepis*; and by Moe (1969) for *E. morio*. More recently, the criteria proposed by Sadovy and Shapiro (1987) have been applied to confirm functional hermaphroditism in *E. guttatus* from Puerto Rico (Shapiro *et al.*, 1993), in *M. bonaci* from the Florida Keys and Florida's Gulf coast (Crabtree and Bullock, 1998), and in *M. microlepis* from the eastern and northeastern Gulf of Mexico (Collins *et al.*, 1987, 1998; Hood and Schlieder, 1992; Koenig *et al.*, 1996). In Cuba, García-Cagide *et al.* (1999) identified three transitional *M. tigris* individuals, confirming protogyny in this species. The present results confirm the diagnosis of functional protogynous hermaphroditism for the species *E. morio* and *M. venenosa*, and for populations of *E. guttatus*, *M. bonaci* and *M. tigris* from the southern Gulf of Mexico. Although protogyny has been confirmed for *M. microlepis* in previous studies, distinctive transitional individuals were not identified among the studied specimens from the *M. microlepis* population of Campeche Bank, probably because the total analysed offshore sample size for this species was too small (N = 212). Normally, relatively small numbers of transitional gonads are observed in grouper species from the Gulf of Mexico and Caribbean Sea such as *E. guttatus* (Shapiro *et al.*, 1993), *E. morio* (Moe, 1969; Collins *et al.*, 2002), *M. bonaci* (Crabtree and Bullock, 1998), and *M. microlepis* (Collins *et al.*, 1987; Hood and Schlieder, 1992; Koenig *et al.*, 1996). This is also the case for *Epinephelus marginatus* (Lowe, 1834) in the south-western Atlantic (Condini *et al.*, 2014). Notwithstanding, higher rates of transitional individuals were observed for other groupers as *Epinephelus aeneus* (E. Geoffroy Saint-Hilaire, 1917) (6.1%) and *Epinephelus guaza* (= *E. marginatus* Lowe, 1834) (2.2%) from the Mediterranean Sea (Buslé and Buslé, 1975), and *Epinephelus microdon* (= *E. polyphekadion* Bleeker, 1849) (6%) from the Tuamotus (French Polynesia) (Buslé *et al.*, 1992). One explication for the low frequency of transitional individuals in field collections is that sex change in groupers is probably completed rapidly. Another possibility is that transitional individuals are not detected because male/female tissue distribution within the hermaphrodite gonad may be along a posterior-anterior gradient in some individuals. If histological sections were not taken throughout each gonad, the transitional condition would be missed under these circumstances. However, this kind of gonadal structure is never seen in groupers; indeed, their germinal tissue configuration is of the undelimited type in which male and female tissues are not topographically separated but intermixed throughout the entire gonad during sex reversal (Shapiro, 1987; Sadovy and Shapiro, 1987).

Sexual transition in groupers from Campeche Bank was observed during breeding and nonbreeding seasons in all species, except *M. venenosa*, for which transitional individuals were recorded only during the nonbreeding season. In offshore waters of Campeche Bank, spawning peaks for *E. morio*, *M. bonaci* and *M. microlepis* occur between January and March (Brulé *et al.*, 1999, 2003a, b). At Arrecife Alacranes and Bajos del Norte, *E. guttatus* forms spawning aggregations between February and March. At Bajos del Norte, *M. tigris* forms aggregations from April-June and *M. venenosa* from March-May (Caballero-Arango, 2013; Caballero-Arango *et al.*, 2013; Tuz-Sulub and Brulé, 2015).

Protogynous hermaphroditism in the studied grouper populations causes bimodal size-frequency distributions, with males larger than females, and an overall sex ratio biased in favour of females in most species. The asymptomatic sex ratio exhibited by *E. guttatus* at both reefs, and by *M. tigris* at Bajos del Norte, may result from differential movement of males and females into and out of spawning aggregations (Sadovy *et al.*, 1994; Nemeth *et al.*, 2007; Kadison *et al.*, 2009). Size selectivity in fishing methods may also explain male dominance in collections (Sadovy *et al.*, 1994).

In many protogynous groupers, lack of clear morphological distinction in testicular structure between primary and secondary males precludes distinguishing between the two male developmental pathways (Sadovy de Mitcheson and Liu, 2008). Most of the testes analysed here retained characteristics of a previous female phase, such as an ovarian-like membrane-lined central cavity and a lamellar structure, as well as developed sperm sinuses in the gonadal capsule. However, the examined males may not necessarily correspond to a secondary male stage since some, called functional primary male analogues (Robertson *et al.*, 1982), may not have previously passed through a functional female phase (prematurational sex reversal situation; Sadovy and Shapiro, 1987). Nonetheless, absence of primary males or bisexual individuals in *E. morio*, *M. bonaci* and *M. microlepis* juveniles caught in inshore waters suggests that the Campeche Bank populations of these three species may be monandric. The scarcity of small juvenile individuals of *E. guttatus*, *M. tigris* and *M. venenosa* in collections taken at

Arrecife Alacranes and Bajos del Norte prevents any speculation about their monandry or diandry. Clearly distinguishing between the two male developmental pathways in these three grouper species will require histological examination of a much wider size range of juvenile specimens.

Acknowledgments. – This research was funded by European Community (EC) grant C1*0432 ME(JR), the Science and Technology Council of the French Embassy in Mexico (CST) grant P 65000 ME, and the Science and Technology Council of Mexico (CONACYT) grants 2184P-B9507, 37606-B; 49963/2411 and 225614. Collections were made possible through the SEMARNAP/S.S.S. 24 de Febrero/CINVESTAV agreement for use of the fishing vessel *UNICAP VII*, and were authorized with fishing license No. 0330400-213-03 from the SEMARNAP. Collections at Arrecife Alacranes and Bajos del Norte were authorized by fishing licenses DGOPA.04608.090508.1131 and DGOPA.08046.270709.2431 from the SAGARPA/CONAPESCA. We thank two anonymous reviewers for providing helpful suggestions.

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